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Pathway analysis of a genome-wide gene by air pollution interaction study in asthmatic children

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Abstract

Objectives: We aimed to investigate the role of genetics in the respiratory response of asthmatic children to air pollution, with a genome-wide level analysis of gene by nitrogen dioxide (NO_2) and carbon monoxide (CO) interaction on lung function and to identify biological pathways involved.

Methods: We used a two-step method for fast linear mixed model computations for genome-wide association studies, exploring whether variants modify the longitudinal relationship between 4-month average pollution and post-bronchodilator FEV_1 in 522 Caucasian and 88 African-American asthmatic children. Top hits were confirmed with classic linear mixed-effect models. We used the improved gene-set enrichment analysis for GWAS (*i-GSEA4GWAS*) to identify plausible pathways.

Results: Two SNPs near the *EPHA3* (rs13090972, rs958144) and one in *TXNDC8* (rs7041938) showed significant interactions with NO_2 in Caucasians but we did not replicate this locus in African-Americans. SNP-CO interactions did not reach genome-wide significance. The *i-GSEA4GWAS* showed a pathway linked to the HO-1/CO system to be associated with CO-related FEV_1 changes. For NO_2-related FEV_1 responses, we identified pathways involved in cellular adhesion, oxidative stress, inflammation, and metabolic responses.

Conclusion: The host lung function response to long-term exposure to pollution is linked to genes involved in cellular adhesion, oxidative stress, inflammatory and metabolic pathways.

Keywords

air pollution; asthma; genome-wide; gene-environment interaction; lung function; pathways

Introduction

Epidemiological studies have demonstrated a strong association between exposure to ambient air pollution and adverse effects on childhood respiratory health[1–3], with asthmatic children being more susceptible to the negative effects of air pollution[4–6]. Lower lung function levels in asthmatic and non-asthmatic children have been associated with short-term exposure to air pollution[3, 7, 8], but the long-term effects of pollution on lung function are less well studied in asthmatic children[9–12].

Known biological mechanisms by which air pollution can impair health include autonomic dysfunction, oxidative stress, and systemic inflammatory responses[13–16]. Respiratory response to air pollution varies between individuals suggesting that genetic susceptibility likely plays a role[17]. Recent genome-wide interaction analyses of chronic air pollution exposure indicated that gene-environment interactions are important for asthma development[18] and for lung function decline in non-asthmatic adults[19].

In asthma, also genes play a role in determining the susceptibility to the harmful effects of air pollution[20] but the underlying biological mechanisms of air pollution-mediated health effects are not fully understood warranting further examination of the genes and pathways that might be involved.

We previously investigated the longitudinal relationship between the 4-month average exposure to air pollution and post bronchodilator (BD) forced expiratory volume in 1 second (FEV_1) and showed that among the measured air pollutants, long-term exposures to carbon monoxide (CO) and nitrogen dioxide (NO_2) are associated with reduced levels of FEV_1 in children with asthma[21]. In the current study we use a hypothesis-free, genome-wide analysis to investigate whether genetic variants modify the long-term effects of CO and NO_2 on lung function in children with asthma, and with a pathway analysis we explore further plausible underlying biological pathways of CO and NO_2 mediated effects on lung function in asthmatic children.

Materials and Methods

The Childhood Asthma Management Program (CAMP; ClinicalTrials.gov Identifier: NCT00000575) study design and methods have been described elsewhere[22]. Additional details on all methods used in the present report are provided in an online data supplement. In summary, children enrolled in CAMP were 5–12 years of age and had airway hyper-responsiveness to methacholine at study entry. 1,041 children entered the randomization phase and 311, 312, 418 children received budesonide, nedocromil, and placebo, respectively. All subjects were treated and followed for four years with visits at two and four months after randomization and at four-month intervals thereafter. Each parent or guardian signed a consent form and participants of 7 years of age and older signed an assent form approved by each clinical center’s institutional review board.

Spirometry before and after the administration of two puffs of albuterol (bronchodilator) was conducted at randomization (RZ) and at follow up visits (n=13) according to the American Thoracic Society Standards[23]. Twenty-four hour average concentrations of CO and NO_2 were estimated for each metropolitan area using data from the United States Environmental Protection Agency’s Atmospheric Integrated Research Monitoring Network. The ZIP or postal code centroid coordinates were used to link participants to daily concentrations from the nearest monitor within 50 km that did not have missing data on that day (December 1993 through June 1999). Averaging the daily pollution concentrations for the 4-month intervals between the clinic visits for lung function measurement created the moving averages.

Genome-wide single nucleotide polymorphisms (SNP) genotyping for CAMP subjects (their families and iControlDB controls) was performed on Illumina’s HumanHap550 Genotyping BeadChip (Illumina, Inc., San Diego, CA).

Statistical Analysis

Genome-wide interaction study—In a genome-wide interaction analysis the computational effort needed to evaluate the effects of hundreds of thousands SNPs on the longitudinally measured trait is prohibitively large with a classic linear mixed model (LMM) approach. We followed the Sikorska et al. conditional two-step approach for fast linear mixed model computations for genome-wide association studies (GWAS)[24], a method to explore whether the longitudinal relationship between 4-month averaged pollution (CO and NO_2) and post-BD FEV_1 %predicted is modified by SNPs in the human genome. The practical application of this approach is to be used as a surrogate of classic LMM, hence we performed the genome-wide scan for hundreds of thousands SNPs in a fast manner.

In summary, in the first step we fitted a LMM with subject-specific (random) intercept and slope for pollution exposure with all SNP terms omitted (main effect and interaction with pollutant) from the model. LMM tests were performed in the R programming language (version 3.5.0 (2018–04-23)), and code availability can be requested by the corresponding author.

At the second step, simple linear regression tests of SNPs genome-wide with an individual's FEV_1 response to CO and NO_2 (subject-specific-random slopes of pollution as given by LMM in step 1), respectively were performed in PLINK[25], using an additive allelic model. SNPs included in the genome-wide analysis had a minor allele frequency > 5% (n=474,792).

For estimating the exact effect size of the interactions and confirm statistical significance, top signals for SNP-pollution interaction as given by 2-step approach (*P*-value $<10^{-5}$) were tested with the classic LMM including terms of pollution, SNP and SNP-pollution interaction, (e.g., Bonferroni corrected minimally significant *P*-value being 0.05/474,792=1.05E-07). Non-Hispanic white (Caucasian) CAMP subjects (n=522) were used as the primary study population and African-American CAMP subjects (n=88) served as the replication study population.

Pathway-level analysis for the genome-wide SNP by pollutant interaction analysis—To analyze pathway-level SNP- pollutant interactions we used the improved gene-set enrichment analysis for GWAS (*i-GSEA4GWAS*; http://gsea4gwas.psych.ac.cn/inputPage.jsp)[26], GSEA evaluates whether the distribution of genes sharing a biochemical or cellular function is different from the distribution of a ranked genome-wide gene list[26, 27]. Details on the *i-GSEA4GWAS* method are given in the supplementary material.

Input data to perform the pathway-level analysis of the SNP-pollution interaction analysis were *P*-values of the two-step genome-wide SNP-pollution interaction analysis in Caucasian CAMP subjects. We changed the default settings and selected specific parameters for the gene-set enrichment analysis; to avoid overrepresentation of SNPs in more than one gene we restricted mapping SNPs to +/−20kb around a gene. We selected additional filtering for gene set size, set to at least 5 genes, so any narrow functional categories would not be missed. Next, the default canonical pathway method of gene-sets was used for further analysis. These canonical pathways were extracted and curated from Molecular Signatures Database from a variety of online resources (MSigDB v2.5; http://www.broadinstitute.org/gsea/msigdb/). The genome-wide *P*-values were transformed to –log (*P*-values), represented genes were mapped based on SNPs P-values, and the enrichment score was calculated.

Significant genes in a pathway are defined as the genes mapped with at least one of the top 5% *P*-values of all SNPs (0.05* 474,792 = 23,740 SNPs). Each significant gene was represented by the SNP in that gene with the lowest genome-wide SNP-pollutant interaction *P*-value (top SNP per significant gene). We selected the top SNPs of all given pathways and with classic LMM we estimated the interaction effect size for the gene-sets most significant SNP-pollutant interactions in Caucasians.

Results

All subjects in CAMP considered in this analysis were randomized and followed up during the trial period. A total of 1,003 of the 1,041 randomized children (96.3%) had pollution data available of which 610 were studied in the genetic analysis. At study entry the mean (SD) age was 9 (2.1) and geometric mean (min-max) PC_{20} 1.1 (0.02–2.5) mg/ml. Table 1 shows the main characteristics of the participants. 82.5% of the children attended all visits during the 4-year trial (median number of completed visits=14 (range: 1–14)). Each participant had a median of 10 (range: 1–10) post-BD lung function measurements. Repeated FEV_1 measurements increase the power of our statistical analysis to detect significant differences between means (8200 and 8600 observations for NO_2 and CO analysis, respectively). Tables S1-S2 summarizes the 4-month moving averages pollutant concentrations during December'93-June'99, with number of observations, percentiles and interquartile range (IQR). CO and NO_2 were weakly correlated (spearman rho=0.30).

Two-step genome-wide SNP by pollutant interaction analysis

Figure 1 presents an overview of our study design and results of the GWIS. After MAF pruning, 474,792 SNPs were included in the primary analysis, and the smallest *P*-values for SNP-NO_2 and SNP-CO interactions with the 2-step approach were 1.37E-06 and 2.04E-06 respectively, showing only suggestive evidence for genome-wide SNP-pollutant interactions (Table 2 and tables S3A and S4). The quantile-quantile (QQ) plots of the two-step GWIS are presented in Figures S1-S2, showing that the distribution of association *P*-values was similar to that expected for a null distribution and that no *P*-values met the conventional genome-wide statistically significant levels (e.g., Bonferroni corrected minimally significant *P*-value being 0.05/474,792=1.05E-07; see Figures S1 and S2).

Confirmation by classic linear mixed model testing

We selected the six top signals (*P*-value $<10^{-5}$) SNP-NO_2 interactions given by the two-step approach and with the classic LMM model we assessed the effect size of these interactions and compared their *P*-value as given by the two approaches. In Caucasians, change in post-BD FEV_1 %predicted per IQR increase in NO_2 level ranged from −1.3 to 1.1 for the 6 SNP-NO_2 interactions. With the classic LMM model the *P*-values decreased for 5 out of 6 SNP-NO_2 interactions with values ranging from 1.3E-08 to 8.5E-06 (table S3A). Three SNP-NO_2 interactions reached genome-wide significance with the classic LMM: rs13090972 (80kb 5' of EPHA3) and rs958144 (162kb 5' of EPHA3) near *EPHA3* (LD between 2 SNPs r^2=0.55) and rs7041938 in *TXNDC8* – the latter in high linkage disequilibrium (r^2=0.8) with rs12684188 in *SVEP1* (Table 2). Similarly, in African Americans the *P*-values of associations were lower with LMM, but none reached genome-wide statistical significance (all *P*-values > 0.05; see table S3B). Table S4 shows that the seven top signals (*P*-value <10–5) SNP-CO interactions as given by the two-step approach did not reach genome-wide statistical significance with LMM. The change in post-BD FEV_1 %predicted per IQR increase in CO level ranged from −0.98 to 0.83 and *P*-values range from 9.69E-07 to 1.26E-05.

Pathway-level analysis for the two-step genome-wide SNP by pollutant interaction analysis on FEV_1 %predicted

For the *i-GSEA4GWAS* in Caucasian CAMP subjects, -log (*P*-values) of 474,792 gene variants were imported and 265,485 variants were mapped on genes +/−20kb (total number of genes: 16,854). We identified one pathway interacting with CO (*P*-value=0.001) and 23 pathways interacting with NO_2 (*P*-values: 0.0001–0.01). Table S5 presents the *i-GSEA4GWAS* suggested pathways for the two pollutants. Details for each individual pathway (SNPs, mapped genes, gene sets, FDR, *P*-value, description) of NO_2 and CO mediated effects can be found http://gsea4gwas.psych.ac.cn/getResult.do?result=13F3A972887892430E6A5C369D76FEAD_1372283303807 and http://gsea4gwas.psych.ac.cn/getResult.do?result=13F3A972887892430E6A5C369D76FEAD_1372284527739, respectively. All the pathways we present in our findings had FDR<0.25. In summary, the *i-GSEA4GWAS* showed a pathway (PAC1R; receptor of pituitary adenylate cyclase-activating polypeptide (PACAP)) to be associated with CO-related FEV_1 changes. For NO_2-related FEV_1 responses, we identified several pathways involved in inflammation, oxidative stress, the HO-1/CO system, calcium homeostasis, cellular adhesion and metabolic responses.

Within each gene-set/pathway there were significant genes (genes mapped with at least one of the top 5% of all SNPs-pollutant interactions in the 2-step genome-wide analysis). Each significant gene is represented by the SNP in that gene with the lowest genome-wide *P*-value of SNP by pollutant interaction (the top SNP per significant gene). Effect sizes of interaction of those SNPs with pollutants as given by LMM are shown in the supplementary material (see tables S6 and S7).

Discussion

Most gene–air pollution studies have focused on a few candidate genetic variations and investigated short-term exposures to pollution[17]. Although these small hypothesis-driven studies can contribute to our understanding of specific gene–pollution effects, they often fail to uncover novel disease-causing mechanisms and in some cases have not been replicated by subsequent studies[28, 29]. To the best of our knowledge, this is the first longitudinal GWIS on lung function response to ambient air pollution in asthmatic children. We used the 2-step approach as a screening tool to identify genes that may interact with air pollution while gaining computational time, and we confirmed the top hits of the 2-step approach with the classic LMM; we used the genome-wide output for a *iGSEA4GWAS*. Below we discuss the putative genes involved in air pollution effects on lung function in childhood asthma and the identified pathways.

At SNP-level, two loci, the *EPHA3* (receptor tyrosine kinase of Eph family; location 3p11.2) and *TXNDC8* (thioredoxin domain containing 8 (spermatozoa) or Spermatocyte/Spermatid-Specific Thioredoxin-3; location 9q31.3) genes showed genome-wide statistical evidence for interaction with NO_2 (with the classic LMM). The best-documented function of the

Eph-receptor/ephrin-A signaling is the regulation of cell adhesion and migration processes critical for a wide variety of including tissue remodeling and immune surveillance[30, 31]. Recent findings suggest that Eph-signaling is involved in pathological conditions such as lung cancer, yet its role in asthma is unknown[32, 33]. The fact that receptor tyrosine kinase pathways contribute to aspects of airway inflammation, airway hyper-responsiveness and remodeling of asthma[34], suggests that we may have identified a novel receptor tyrosine kinases (EPHA3) important for the pathogenesis of asthma in response to NO_2 in Caucasian children.

The second top signal locus, *TXNDC8*, belongs to the thioredoxin reductase enzymes, a well-characterized subfamily of selenoproteins that perform an essential redox role in immune cells[35]. Recent studies indicated that Thioredoxin system may contribute to the pathogenesis of COPD, asthma and lung injury and suggest that this pathway may be used in future therapeutic applications[36]. The genome-wide top hit SNP (rs7041938) in *TXNDC8* found to modify the NO_2 effects on FEV_1 in Caucasian subjects is in high linkage disequilibrium (r^2>0.8) with rs12684188 in *SVEP1* (sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1; location 9q32). In a recent GWAS, a locus containing the *SVEP1* gene showed signals of association with FEV_1 decline in non-asthmatic adults[37]. In our asthmatic children the interaction *P*-value of the *SVEP1* variant did not reach significance. *SVEP1* codes for a protein called polydom, which is recognized as a cell adhesion molecule with a biological role in cellular adhesion and/or in the immune system[38, 39]; but its role in asthma has not been investigated so far. We were unable to replicate these loci in African-Americans and it would be important to replicate our finding in other populations in the future.

The pathway analysis helps to clarify biological plausible connections for our GWAS hits with one another. Some of the pathways identified from our *iGSEA4GWAS* analysis have been previously found to play a role in asthma and be related to cellular adhesion and immune response, as do so our GWAS top loci. The first genome-wide gene by interaction study on asthma development identified genes involved in glycosphingolipids biosynthesis, G-protein coupled receptor signalling and adhesion[18]. Similarly, our *iGSEA4GWAS* identified sphingolipid (glycosphingolipid metabolism pathway), G-coupled receptor (gs-pathway, agpcr-pathway, plce-pathway) and epithelial adhesion (HSA04514 cell adhesion molecules pathway) pathways in lung function response to NO_2, pointing to the same direction. Sphingolipids and altered sphingolipid metabolism have emerged as potential key contributors to the pathogenesis of asthma[40]. Orosomucoid-like 3 gene (*ORMDL3*) and the asthma susceptibility locus 17q21 have been strongly and reproducibly linked to childhood asthma[41].

The role of airway epithelial barrier function (HSA04514 cell adhesion molecules pathway) in the susceptibility to develop allergic asthma has been extensively studied and polymorphisms in adhesion molecules genes have been associated with asthma and asthma severity[42–44]. It is plausible that exposure to NO_2 induces oxidative stress with cellular barrier damage and inflammatory responses. In the supplementary material we describe in more detail how pathways involved in inflammation and oxidative stress (NOS1, HSP27, IL10, Heme biosynthase) may be linked to NO_2 exposure and how they are inter-related.

Metabolic pathways (feeder of glycolysis and obesity pathways) are activated to compensate the cellular demands to stress and the HO-1/CO system may protect against oxidative stress and inflammation. In line with our findings, a GWIS study of non-asthmatic adults, identified a mechanistic link between adiponectin (a metabolic biomarker with modulating action on inflammatory processes systemically and locally in the lung) and cadherin 13 as a biologically plausible pathway for modifying the air pollution exposure effect on lung decline[19].

Oxidative stress has been associated with calcium influx regulation, two responses observed in our pathway analysis as well[45, 46]. Interestingly, a proteomic-based study has shown that allergen-induced early asthma response in rats is associated with glycolysis, calcium binding and mitochondrial activity[47], supporting our identified underlying molecular mechanisms for response to environmental toxicants in asthma.

The *iGSEA4GWAS* of CO interactions suggested the neuropeptide pituitary adenylate cyclase-activating peptide receptor (PAC1R) pathway in CO-related response. The ligand of PAC1R (PACAP) can induce bronchodilation and endogenous regulation of airway tone by means of a CO-dependent mechanism with local HO-1/CO release in the airway smooth muscle, and it also has pro-inflammatory functions that require calcium regulation[48–50]. Furthermore, PACAP, acting through type 1 PACAP receptor, exerts a potent protective effect against oxidative stress-induced apoptosis[51].

Our childhood asthma study had the advantage of having a long follow-up period with high attendance of the subjects and repeated lung function measurements, air pollution levels during that period and genomic data. The two-step approached used for longitudinal data[24] provided shorter processing time and we confirmed its accuracy, i.e., at a second stage the genome-wide top signals found by the two-step approach were confirmed by LMM testing.

We could not find a second study of asthmatic children with similar design, repeated lung function measurements, population characteristics, genome-wide genotyping and air pollution data. Although population stratification is less likely to bias estimates of gene-environment interaction effects[52], we used as our primary study only Caucasian CAMP subjects and found no evidence of stratification in our Q/Q plots. For replication studies, definition and measurement of the exposure and/or outcome is critical to the success of gene–environment investigations, therefore we decided to use the second largest ethnic subgroup of the CAMP as our replication population (although of relative small size), to ensure that the genotyping, outcome and exposure were measured reliably and consistently. This reduced power and potential different linkage disequilibrium patterns in the replication population represent limitations of this study.

After testing for pollution effect modification at the SNP-level, we performed the pathway approach to assess the overall evidence of interaction of pollution with a group of functionally related genes, thus incorporating prior biological knowledge. Our pathway-level analysis of SNP-pollution interactions identified biological plausible mechanisms for pollution-mediated asthma progression in children that are generally consistent with the SNP-level analysis.

Our findings highlight the promise of pursuing genome-wide gene-environment interaction studies in smaller populations with high quality longitudinal exposure information by showing that they can identify biologically relevant effects of these exposures. We conclude that genetic susceptibility to traffic-related air pollutants such as with CO and NO_2 are linked to oxidative stress and inflammation pathways, while metabolic pathways including calcium homeostasis and the HO-1/CO pathway may play a cytoprotective role against oxidative stress and inflammation. Our findings may represent the first step for functional research and pharmacological developments for protection against the detrimental effects of air pollution on asthma severity and progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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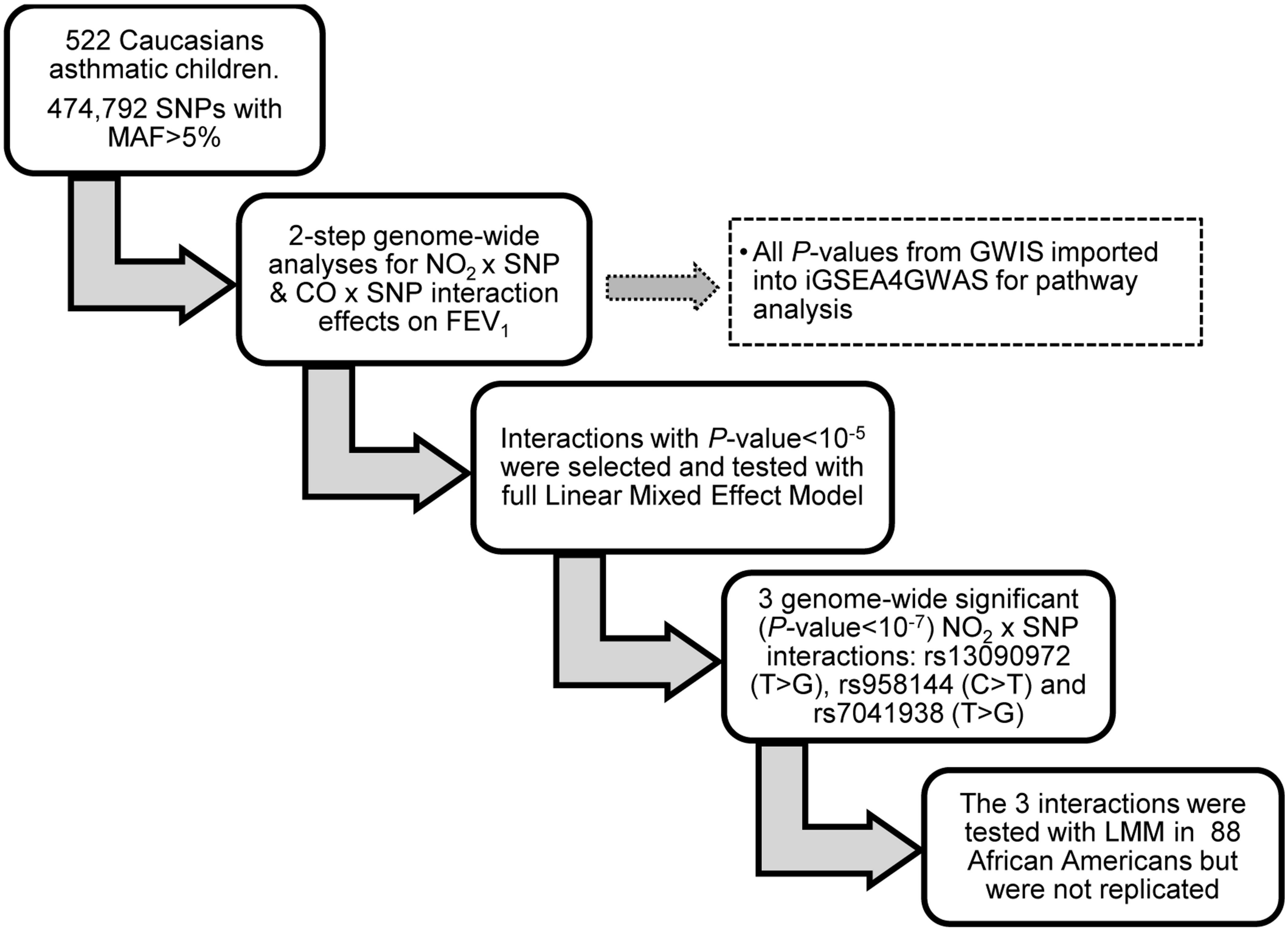

Figure 1.

presents the flow chart with the analytic steps and summary of results of the genome-wide gene by pollutant(s) interaction study. Top hit SNPs ($P<10^{-5}$) interacting with pollutants in Caucasians were selected and with LMM we assessed the interaction effect size and p-values. Genome-wide significant interactions ($P<10^{-7}$) were tested for replication in African-Americans.

CO: carbon monoxide; NO_2: nitrogen dioxide; LMM: linear mixed model; SNP: single nucleotide polymorphism, MAF: minor allele frequency; GWAS: genome wide interaction study; iGSEA4GWAS: improved gene-set enrichment analyses for GWAS; FEV_1: forces expiratory volume in 1 second

Table 1.

Population characteristics

	N= 1003
City; n (%)	
Albuquerque	121 (12.1)
Baltimore	126 (12.6)
Boston	123 (12.3)
Denver	141 (14.1)
San Diego	122 (12.2)
Seattle	136 (13.6)
Saint Louis	133 (13.3)
Toronto	101 (10.1)
Sex; n (%)	
Males	602 (60)
Females	401 (40)
Treatment Group; n (%)	
Placebo	407 (40.6)
Budesonide	298 (29.7)
Nedocromil	298 (29.7)
Ethnicity; n (%)	
Caucasians	677 (67.5)
African-Americans	137 (13.7)
Hispanics	97 (9.7)
Other	92 (9.2)
Annual Income ≥30K USD; n (%)	
Yes	728 (76)
No	235 (24)
In utero smoking exposure; n (%)	
Yes	114 (14)
No	854 (86)
Pre bronchodilator lung function at randomization; mean (SD)	
FEV_1%predicted	93.8 (14.3)
FVC %predicted	104.0 (13.1)
FEV_1/FVC%	79.7 (8.3)
Post bronchodilator lung function at randomization; mean (SD)	
FEV_1%predicted	103.0 (12.8)
FVC %predicted	106.5 (12.8)
FEV_1/FVC%	85.5 (6.5)

FEV_1 : forced expiratory volume in 1 second; FVC: forced vital capacity; SD: standard deviation; =>30K USD: equal or more than 30,000 United State Dollars

Table 2:

Top genome-wide gene by nitrogen dioxide interaction loci and suggested functions

Top genome-wide interaction locus	SNP	2-Step approach *P-values*	Classic LMM *P value*	LMM Change per IQR	Function(s) related to genes[a]	Identified pathways[b] linked to those gene functions
Near EPHA3;	rs13090972 (T>G)	1.37E-06	**1.33E-08**[*]	-1.33	**Receptor tyrosine kinase of Eph family** Cell adhesion; Immune surveillance; Tissue remodeling	Cell adhesion molecules; Calcium regulation; Glycosphingolipids metabolism, Glycosaminoglycan (chondroitin) biosynthesis
chr3:88994672 and 89076896	rs958144 (C>T)	4.81E-06	**2.94E-08**[*]	−1.28		
TXNDC8; chr9:113091523	rs7041938 (T>G)	7.35E-06	**1.04E-07**[*]	1.14	**Thioredoxin reductase family** Cell redox homeostasis	HSP27, iNOS, IL10, Heme biosynthesis–Heme oxyganase-1/CO, Calcium regulation
in LD=0.8 with SEVP1; chr9:113133588	rs12684188 (C>T)	> 1E-05	3.88E-07	1.20	Cell adhesion; Immune surveillance	Cell adhesion molecules, Glycosphingolipids metabolism, Calcium regulation

SNP: single nucleotide polymormpism, LMM: linear mixed model, IQR: interquartile range of 4-month average nitrogen dioxide concentration (4 parts per billion).

[*] genome-wide significance (P< 1.05E-07)

[a] based on coding protein’s function(s). Details for each gene are given in the text .

[b] based on pathway analysis